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Effect of Laser Pulse Repetition Rate and Pulse Duration on Mast Cell Number and Degranulation

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Background and Objective: **Mast cell activation by low-level laser therapy (LLLT), leading to degranulation and the release of mediators, may be one of the mechanisms by which LLLT can accelerate tissue repair in mammals. The objective of this work, part of an investigation to determine the optimum parameters for increasing mast cell number and degranulation in injured skin, was to determine the effect of different pulsing frequencies of LLLT.**

Study Design/Materials and Methods: **Partial-thickness wounds in anaesthetized adult male Wistar rats were irradiated immediately after injury with monochromatic coherent light (wavelength 820 nm) pulsed at either 2.5, 20, 292, or 20,000 Hz at an average power density of 800 mW/cm^2 for 27 seconds; the energy density was 21.6 J/cm^2. The effects on mast cell number and degranulation were assessed 2 hours post-treatment by counting the numbers of intact and degranulated mast cells in Carnoy-fixed, toluidine blue-stained, sections of irradiated and sham-irradiated wounds.**

Results: **The total number of mast cells was increased significantly ($P<0.05$) by all the frequencies when compared to the sham-irradiated group, but there was no significant difference between frequencies ($P>0.05$). However, although the number of degranulated mast cells was higher in all laser-treated wounds, in comparison with the sham-irradiated group, only the 20 Hz (pulse duration 45 ms) and 292 Hz (pulse duration 3 ms) frequencies were significantly effective ($P<0.05$).**

Conclusion: **Increase in mast cell number is not pulsing frequency dependent, whereas degranulation is.**

Key words: Carnoy's solution, frequency, partial thickness wound, rat

INTRODUCTION

A number of reports showed that exposure to laser irradiation can accelerate tissue repair in mammals [1–4]. The exact mechanisms are still speculative but mast cell activation leading to the release of mediators of wound healing from them might be one of mechanisms.

The role of mast cells in the three phases of wound healing: the inflammatory reaction, angiogenesis and extracellular-matrix formation, and remodelling has been confirmed by Trabucchi et al. [5].

Mast cells can be regarded as specialized secretory cells which, in the resting state, contain several hunderd granules that are each surrounded by a membrane. The matrix of the granule is largely a heparin-protein complex in which the protein carboxyl groups serve as histamine binding sites. Other biologically active substances

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found in mast cell granules include glucuronidase, acid phosphatases, a variety of proteases, eosinophil chemotactic factor of anaphylaxis (ECF-A), and neutrophil chemotactic factor (NCF) [6], many of which may be involved in the response of the body to injury. Histamine, for example, may in low doses stimulate collagen formation and healing [7], while heparin stimulates the migration of endothelial cells [8] and ECF-A and NCF may modulate the inflammatory process, acting as chemoattractants for cells involved in later phases of the repair process. The degranulation of mast cells would, by releasing these substances, therefore be expected to stimulate tissue repair.

In a previous work, we showed that mast cells can be activated and their total number increased by light at certain wavelengths [9]. To obtain the best response from any physical modality, the optimum energy density, wavelength, and frequency should be used. In the paper referred to above, we determined the most effective wavelengths (from those which were available) which led to the highest increase in the total number of mast cells and the highest percentage of degranulation. This experiment was designed to determine the effect of different pulsing frequencies of laser light on the total number and percentage degranulation of the mast cells.

METHODS

Six rats were used for each group. The animals used in this investigation were adult male Wistar rats, weighing approximately 250 g. The animals were randomized for therapy and were all treated at the same time from wounding. They were anaesthetized with halothane immediately prior to treatment and maintained under general anaesthesia throughout the treatment period. The skin of the right flank of each rat was shaved and cleansed with the detergent and antiseptic agent Hibitane and then a 1 cm^2 partial thickness wound was made manually using a No. 11 scalpel blade, ensuring that the deep part of the dermis (adjacent to the panniculus carnosus) remained intact. The central part of the wound (4 mm^2), which was marked with Indian ink using a template, was either treated with the laser probe without switching on the machine at a distance of 5 mm away (the sham-irradiated control group) or treated with laser by the application of the probe at a distance of 5 mm away from the wound using different frequencies (the test groups).

The light source used was a Biotherapy 2001 machine produced by Omega Laser Systems Ltd (London, UK). A 100 mW power (gallium alluminium arsenide) laser probe was used.

The machine was calibrated immediately before the experiment using a photodyne (Model 350) "flat response" optical power meter positioned 5 mm below the emitting plane of the diode.

Irradiation Parameters

1) Wavelength = 820 ± 5 nm. 2) Duration of treatment = 27 seconds. 3) The average power density = 800 mW/cm^2. 4) The energy density = 21.6 J/cm^2. 5) The pulsing frequencies tested were 2.5, 20, 292, and 20000 Hz. 6) The pulsing durations were, 360, 45, 3, and 0.045 mseconds respectively.

The device was designed to produce a fixed duty cycle (pulsing frequency X pulse duration) of 90% at all pulsing frequencies in order to maintain a constant average power throughout the experiments.

All the rats were killed painlessly after 2 hours by inhalation of a lethal dose of carbon dioxide, and the wounds were removed, together with the underlying panniculus carnosus, fixed, and processed as follows.

Mast Cell Degranulation Assay

All the skin samples were fixed in Carnoy's solution for 2 hours, as described by Strobel et al. [10]. They were then dehydrated, cleared, and the marked central 4 mm^2 area of the wound was embedded in paraffin wax. Sections were cut at 7 μm and were coded to ensure that they were evaluated blindly from this stage onwards and then stained with toluidine blue as described by Smith and Atkinson [11]. This technique is satisfactory for the examination of intact mast cells and is also particularly useful for the preservation of granules shed from the cells into the surrounding tissues. Intact mast cells show a deep blue (orthochromatic) staining, but as degranulation proceeds the granules become increasingly metachromatic, eventually appearing purple-red.

Based upon the degree of degranulation and metachromasia, it is possible to identify three types of mast cells [12]. In this experiment, however, the mast cells were divided into only two types, the intact and the degranulated [9].

Mast Cell Counts

After staining, randomly selected sections were examined by light microscopy, and for each

TABLE 1. The Total Number of Mast Cells per Hundred High Power Fields and Percent Degranulation After Laser Irradiation With Various Frequencies (Means ± SD)

Frequency (Hz)	Total number/100 HPFs[a]	% Degranulation
0(S-I)[b]	522.0 ± 19.0	36.5 ± 2.0
2.5	677.5 ± 120	39.7 ± 1.7
20	655.8 ± 128	44.1 ± 3.3
292	675.5 ± 105	43.2 ± 2.6
20000	663.0 ± 78	39.0 ± 2.2

[a]HPFs = High power fields.
[b]S-I = Sham-irradiated.

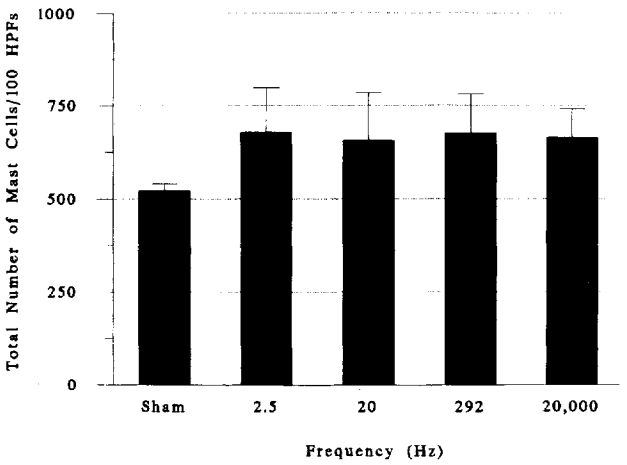

Fig. 1. Effect of various pulse repetition rates of laser on the total number of mast cells in the bed of partial thickness wounds. Sham = Sham-irradiated. Bars are standard errors of the means. HPFs = High power fields.

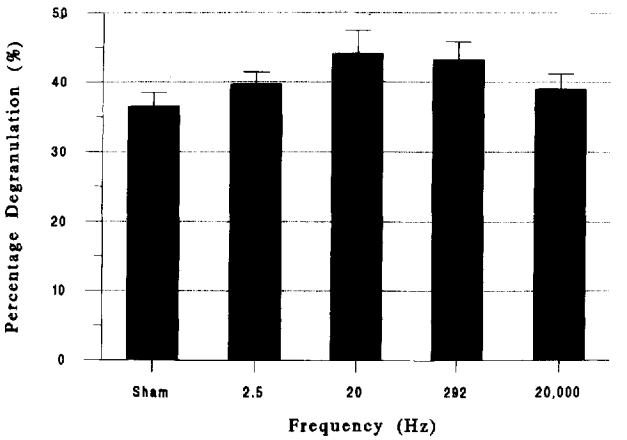

Fig. 2. Effect of various pulse repetition rates of laser on the percentage degranulation of mast cells in the bed of partial thickness wounds. Sham = Sham-irradiated. Bars are standard errors of the means. HPFs = High power fields.

skin sample, the total, the intact, and the degranulated mast cell numbers per 100 high power fields (magnification × 400) were counted. Regions (i.e., high-power fields) where counts were carried out were circular areas of dermis located adjacent to each other immediately superficial to the panniculus carnosus [13]. The strip of the dermis in which counts were made was centered on the midpoint of the part of the wound bed contained in that section. Each region where counts were made had an area of 0.2 mm^2 and the total area examined per specimen was 20 mm^2.

The means of the total number of mast cells, the ratios of the degranulated mast cells and the standard deviations were calculated in the sham-irradiated group, and in each treated group, and compared statistically.

RESULTS

Mast Cell Counts

Mast cells generally appeared blue or purple against a pink background in the sections. The majority of mast cells were located in groups around blood vessels in the deep half of the remaining dermis. The results are summarized in Table 1, and Figures 1 and 2.

Statistical Analysis

Two variables, the total number of mast cells and the ratio of the degranulated mast cells to the total number, were analysed. The distribution of the data was checked for normality using the Shapiro-Francia test. It can be seen from Table 2 that the total number of mast cells is not normally distributed but the ratio is. As a result the non-parametric Jonckheere-Terpstra test was used to investigate the effect of frequency on the total number of mast cells. The probability associated with this was 0.12 indicating no significant effect of frequency on the total number of mast cells. The data for the total number of mast cells is best presented as the median and range for each frequency.

One way analysis of variance was used to analyse the ratio of the degranulated mast cells to the total number of mast cells. Dunnett's multiple comparison of means test was used to compare the ratio at each frequency to the data for the sham. There was a significant effect of the 20 and 292 Hz frequencies on the ratio of the degranulated mast cells.

TABLE 2. Probability Associated With the Shapiro-Francia Test for Normality

Frequency (Hz)	Total	Ratio
0(S-1)[a]	0.96	0.99
2.5	0.08	0.98
20	0.02	0.92
292	0.42	0.42
20000	0.02	0.95

[a]S-1 = Sham-irradiated.

DISCUSSION

It has already been reported that laser irradiation increases the total number and percentage degranulation of mast cells in rodents [9, 14]. In this experiment, the wavelength that produced the greatest effect in an earlier study [9] was selected, but the pulsing frequencies used were varied. The findings of this study showed that laser irradiation at all the frequencies investigated did not increase the total number of mast cells in the wound site, but the percentage of degranulation was frequency dependent. Young et al. [15], examining the effect of light on calcium uptake by macrophages, observed the greatest response when frequencies of between 16 and 36.48 Hz were used, which correlates closely to the findings in this study.

The effect of frequency on the response of the tissues to light was also shown by the observation that in vivo irradiation at an energy density of 3 J at 3000 Hz increasing the rate of healing of experimental wounds in rats, whilst a frequency of 1500 Hz at the same energy density did not do so [16].

Recent work on the effect of light therapy on macrophages showed that exposing cells of the macrophage-like cell line (U-937) in vitro to a 820 nm coherent light source at the following pulsing frequencies: 2.28, 18.24, 292.3, and 1000 Hz, and then adding the conditioned medium to 3T3 fibroblast monolayers, led to the increase in their proliferation especially with the first three frequencies [17]. These findings are also similar to the results of this study.

Cellular effects of exposure to other forms of electromagnetic radiation have also shown both energy intensity and frequency windows, e.g., extremely low frequency magnetic fields [18,19]. The cyclotron resonance theory states that biological effects occur around particular frequencies, and that these depend upon the mass and charge of the particles involved [19]. The major activities of the cell, which are influenced by membrane permeability to ions such as calcium, are modified by these frequency windows. In this experiment, 20 and 292 Hz were the most effective in inducing mast cell degranulation. The effect of the 20 Hz frequency on mast cell degranulation is in agreement with previous findings [15,17]. We thus postulate the presence of a frequency window at or around this frequency and a second window at or around the 292 Hz frequency in mast cells that stimulate their degranulation.

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